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Bacterial diversity in marine hydrocarbon seep sediments

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Summary

Marine seeps introduce significant amounts of hydrocarbons into oceans and create unusual habitats for microfauna and -flora. In the vicinity of chronic seeps, microbes likely exert control on carbon quality entering the marine food chain and, in turn, hydrocarbons could influence microbial community composition and diversity. To determine the effects of seep oil on marine sediment bacterial communities, we collected sediment piston cores within an active marine hydrocarbon seep zone in the Coal Oil Point Seep Field, at a depth of 22 m in the Santa Barbara Channel, California. Cores were taken adjacent to an active seep vent in a hydrocarbon volcano, on the edge of the volcano, and at the periphery of the area of active seepage. Bacterial community profiles were determined by terminal restriction fragment length polymorphisms (TRFLPs) of 16S ribosomal genes that were polymerase chain reaction (PCR)-amplified with eubacterial primers. Sediment carbon content and C/N ratio increased with oil content. Terminal restriction fragment length polymorphisms suggested that bacterial communities varied both with depth into sediments and with oil concentration. Whereas the apparent abundance of several peaks correlated positively with hydrocarbon content, overall bacterial diversity and richness decreased with increasing sediment hydrocarbon content. Sequence analysis of a clone library generated from sediments collected at the periphery of the seep suggested that oil-sensitive species belong to the γ Proteobacteria and Holophaga groups. These sequences were closely related to sequences previously recovered from uncontaminated marine sediments. Our results suggest that seep hydrocarbons exert a strong selective pressure on bacterial communities in marine sediments. This selective pressure could, in turn, control the effects of oil on other biota in the vicinity of marine hydrocarbon seeps.

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Introduction

Nearshore marine hydrocarbon seeps contribute significantly to global hydrocarbon budgets (Hornafius *et al.*, 1999). In the Santa Barbara Channel (SBC) large oil and gas hydrocarbon seeps are a long-term feature, known by missionaries and used by indigenous peoples for centuries (Fisher, 1978). These natural hydrocarbon seeps release hundreds of barrels of oil per day (Allen *et al.*, 1970) and oil and tar balls continually wash up on the beaches of Santa Barbara and the Northern Channel Islands (Elliot, 1999). The Coal Oil Point Seep Field, located a few kilometres offshore from the University of California Santa Barbara (Fig. 1), releases much of this oil and is one of the most active seep areas in the world. From sonar surveys and direct capture, gas seepage estimates range from 10^5 to 2×10^5 m^3 day^{-1} (3×10^{10} and 6×10^{10} g $year^{-1}$) from ~3 km^2 of seafloor (Hornafius *et al.*, 1999; Quigley *et al.*, 1999). Oil seeps from this field at ~80 barrels day^{-1} (Clester *et al.*, 1996). Despite potentially toxic concentrations of hydrocarbons, diverse animal communities inhabit these nearshore seep environments (Spies and Davis, 1979). In fact, it appears that SBC seep oil carbon enters the marine food web (Bauer *et al.*, 1990) and enhances meiofaunal productivity (Steichen *et al.*, 1996). Hydrocarbons enter the food web through meiofauna grazing on the abundant bacteria in marine systems that utilize hydrocarbons as energy sources (Atlas, 1995). While transferring seep carbon to higher trophic levels, seep sediment bacteria also alter the composition (Wenger and Isaksen, 2002) and toxicity (Hamdoun *et al.*, 2002) of seep hydrocarbons. For these roles, and because we typically rely upon naturally occurring microbial populations to remediate oil spills (Head and Swannell, 1999), bacterial communities associated with seeps are of interest.

In general, exposure to defined substrates enriches for

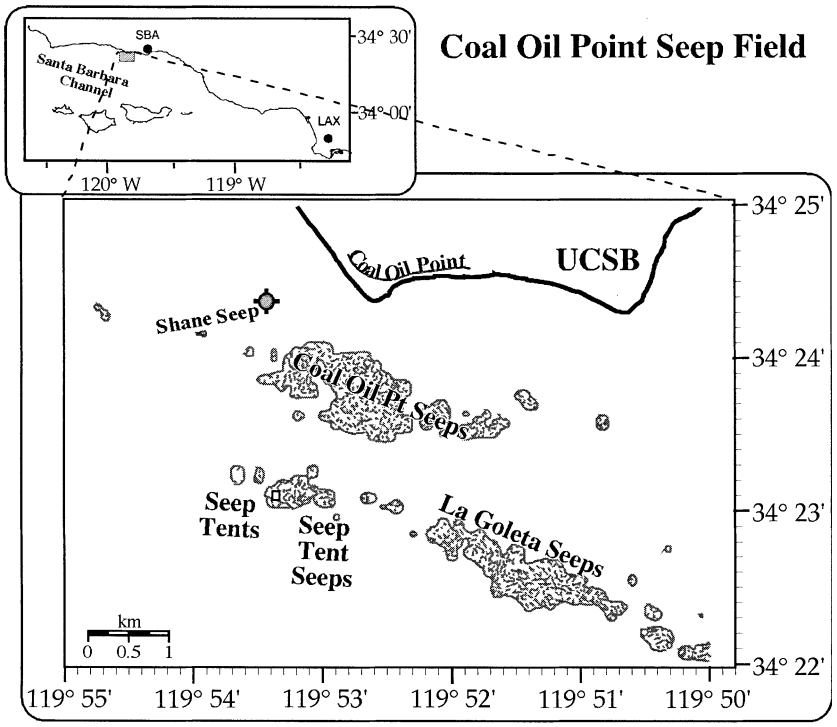

Fig. 1. Coal Oil Point Seep Field.

bacteria capable of utilizing these substrates and thus can control heterotrophic bacterial community structure and metabolic rates (Mills and Mallory, 1987; Kaplan, 2001). Diversity is an important component of bacterial community structure because of the link between diversity and resilience (von Canstein *et al*., 2002). Because of substrate enhancement and toxicity, chemical contaminants usually reduce microbial diversity in natural systems (Trevors, 1998). In systems without previous significant exposure to hydrocarbons, oil spills affect bacterial communities in ways similar to other chemical contaminants: i.e. oil reduces bacterial diversity in soils and sediments (Atlas *et al*., 1991; Lindstrom *et al*., 1999; Macnaughton *et al*., 1999; Delille, 2000; Röling *et al*., 2002). Little is known about bacterial communities persistently exposed to oil, such as in marine hydrocarbon seeps. Although selection, by enrichment and toxicity, should decrease diversity, microbial biomass (Montagna *et al*., 1989) and productivity (Montagna *et al*., 1986) are higher near seeps than surrounding sediments. Macro-ecological species-energy theory (Wright, 1983) predicts that this enhanced biomass corresponds to higher microbial diversity.

To determine the effects of chronically released hydrocarbons on bacterial populations in marine sediments, we examined bacterial community structure and diversity along a radial transect from the centre of an active hydrocarbon seep region. We also examined diversity and community composition as a function of sediment depth because bacterial communities in marine sediments vary significantly with depth (Urakawa *et al*., 2000). We assessed bacterial populations using terminal restriction fragment length polymorphisms (TRFLPs; Liu *et al*., 1997) of 16S rRNA genes that were PCR-amplified with eubacterial primers. Eubacteria dominate microbial communities in marine sediments (Llobet-Brossa *et al*., 1998; Sahm and Berninger, 1998; Ravenschlag *et al*., 2001), hydrocarbon-exposed porous media (Macnaughton *et al*., 1999; Yakimov *et al*., 2002), and presumably marine hydrocarbon seeps. Terminal restriction fragment length polymorphisms are reproducible (Osborn *et al*., 2000) and allow statistical comparisons of highly similar microbial communities (LaMontagne *et al*., 2002). Our analysis of TRFLPs generated from sediments collected in a marine hydrocarbon seep field suggests that chronic exposure to oil reduces bacterial diversity.

Results

Gas chromatography (GC) of total hydrocarbons extracted from sediments collected at Shane Seep revealed a complex mixture of hydrocarbons with few resolvable peaks or low molecular weight compounds (data not shown). Chromatograms generated from Shane Seep appear similar to a previously published chromatogram (Struermer *et al*., 1982) generated from the SBC Coal Oil Point Seep Field. The lack of GC-resolvable peaks and the dominance of the unresolved complex mixture in these chromatograms strongly suggests microbial degradation of the oil (Wang

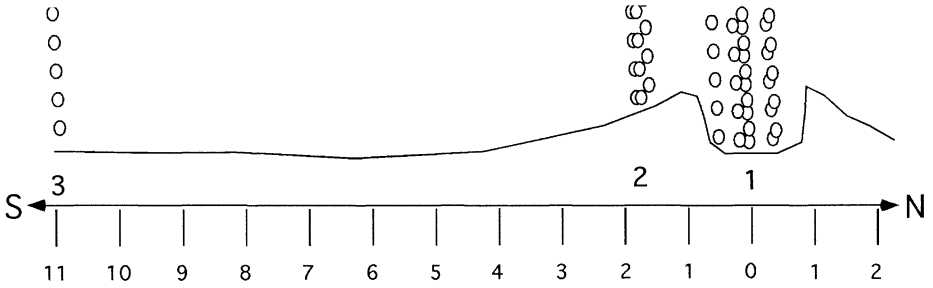

Fig. 2. Schematic of sampling site. Sites (1) centre (2) edge and (3) outer are indicated.

et al., 1998). Gases seeping from sediments in Shane Seep field consist primarily of methane and ethane (Leifer and Clark, 2002) but gas seeping from sediments at an outer site (10 m away from the seep centre) contained fewer alkanes heavier than methane as opposed to gas seeping at the centre site (data not shown). Sediments collected outside the seep region typically contained less than 1% total carbon (g/g dry mass). Sediments collected inside or on the edge on the seep caldera (Fig. 2) contained up to 28% total carbon. Sediment oil and total carbon content correlated (Fig. 3A), which suggests oil seepage supplied the excess carbon. Sediment C/N ratios increased dramatically with oil content reaching an asymptote of ~50 (Fig. 3B). Inorganic carbon contributed to the C/N ratio slightly; however, with increasing oil concentration, the contribution of inorganic carbon is likely to be relatively unimportant.

Terminal restriction fragment length polymorphisms suggested a difference in bacterial community structure due to the presence of oil. For example, peaks observed between 187 and 224 bp in an electropherogram generated from a sample taken at the periphery of the seep area, appeared relatively small or absent in an electropherogram generated from a sample taken at the edge of the main vent (Fig. 4). Sediment depth also influenced community structure. For the three sites (centre, edge and outer) the number of peaks shared between electropherograms tended to be higher for comparisons within depth intervals than between depth intervals but the difference was relatively small. For example, at the centre site electropherograms shared 39 peaks within the surface section and shared 36 peaks between the surface and deep sections (data not shown). Correlation coefficients, based on the heights of shared peaks, also tended to be higher for comparisons within depth intervals than for comparisons between depth intervals. For example, correlation coefficients (R) averaged 0.80 for comparisons between surface sections and 0.73 for comparisons between surface and deep sections. To determine the significance of changes in microbial community structure with depth, we plotted principal components (PCs), derived from Jaccard and Pearson coefficients (see *Experimental procedures*), against depth. The first PC, which explained 28% of the variability in TRFLPs, decreased significantly with depth (Fig. 5A). In terms of this PC, variability between sites was high in surface sediments (0–6 mm), especially at the edge site where sediments contained the most oil. To determine the relationship between hydrocarbon presence and microbial community structure, we recalculated

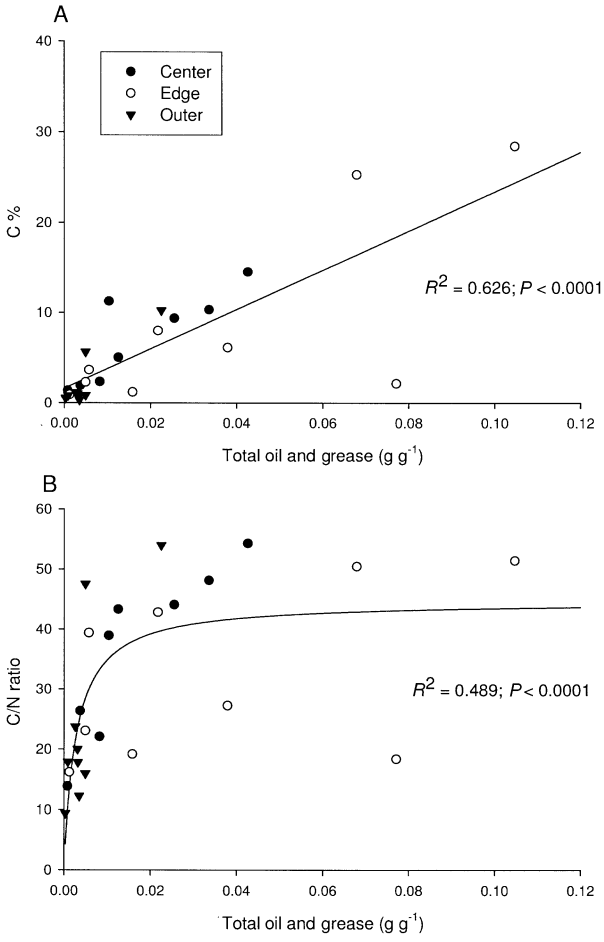

Fig. 3. Sediment carbon and nitrogen content versus total oil. Total oil, carbon (top) and C/N ratio (bottom) were determined as described in *Experimental procedures*. Locations (1) centre (2) edge and (3) outer correspond to locations defined relative to a seep mud volcano (see Fig. 2).

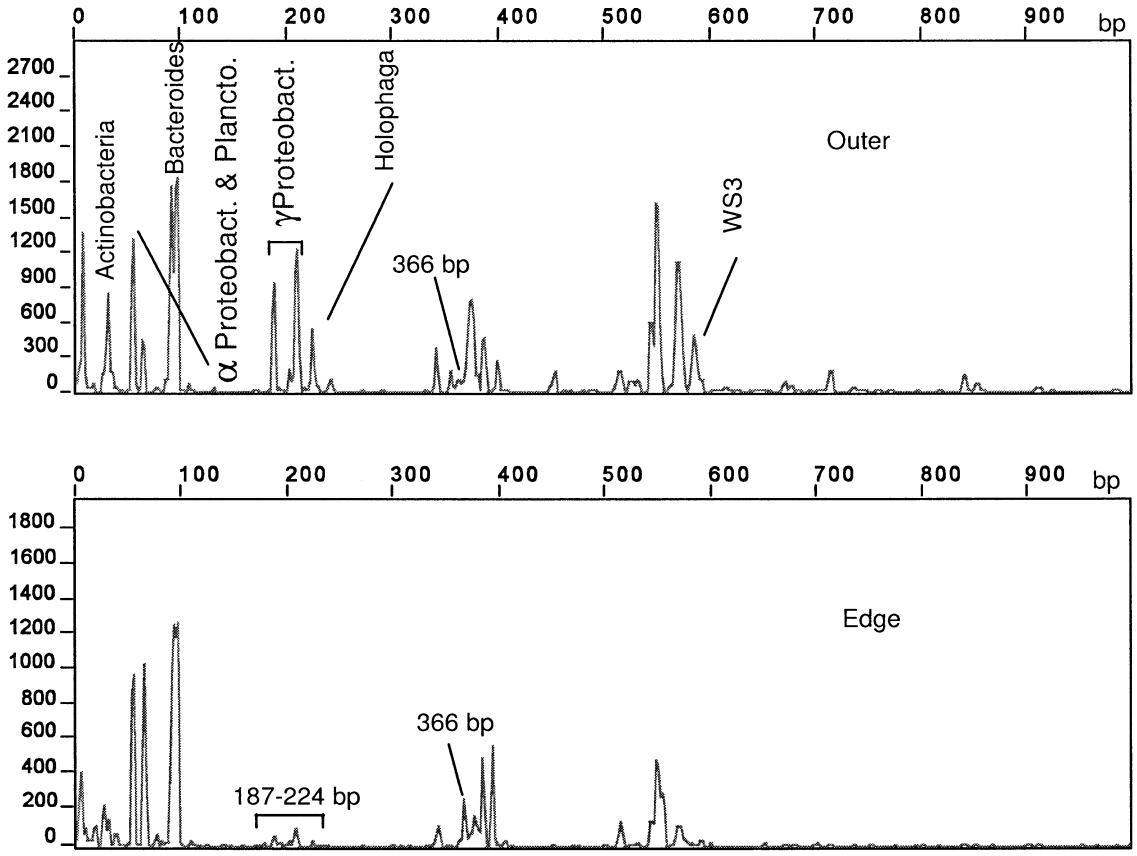

Fig. 4. Typical electropherograms generated by restricting with *Hha* I. Terminal restriction fragments discussed in text are indicated. Relative fluorescence of peaks (*y*-axis) and length of fragments (*x*-axis) are indicated. Outer TRFLPs from surface sediments (4–6 mm) collected at the outer site, where the oil content of sediments was low. Edge TRFLPs from surface sediments (4–6 mm) collected at the edge site, where the oil content was relatively high.

the PC based on the surface sediments (0–6 mm) only. Focusing on this interval minimized changes due to depth and improved the amount of variation explained by the first PC to 68%. This PC correlated well with oil content in surface sediments (Fig. 5B).

Species richness of bacterial communities in surface sediments (0–6 mm), as assessed by TRFLP, decreased with increasing oil content (Fig. 6). Richness, as assessed by the number of replicated peaks, tended to be higher for *Hha* I-generated TRFLPs (averaging 51 ± 5 for *Hha* I versus 43 ± 2 for *Hae* III) but *Hae* III and *Hha* I gave similar diversity estimates (averaging 3.4 ± 0.2). For both enzymes, diversity indices appeared higher at the outer site but the differences between sites were not statistically significant ($P > 0.09$). The statistical overlap in diversity between sites likely reflects the heterogeneity of oil distribution in sediments: oil content, not sample location, predicted diversity (i.e. Fig. 6).

Of the 113 replicated peaks observed in *Hha* I-generated TRFLPs, the height of 14 peaks correlated negatively ($R^2 = 0.197$–0.661; $P < 0.034$) with the oil concentration of the sample. Exponential decay models described the negative relationships between these peak heights and hydrocarbon content (data not shown). This indicates sensitivity to oil for the bacteria that generated these peaks. For six peaks, linear models described positive relationships with hydrocarbon content (data not shown). This suggests that the species that generated these peaks benefited from the presence of oil. The height of a 366 bp fragment in *Hha* I generated TRFLPs (Fig. 4), showed the strongest positive correlation with oil content.

From in silico restriction, we predicted that the oil-sensitive peaks with *Hha* I TRFs between 187 and 224 bp (Fig. 4), most likely corresponded to γ Proteobacteria. We screened a library of cloned rRNA genes generated from the surface samples collected at the 'outside' site (see *Experimental procedures*) for clones with sequences consistent with these restriction patterns. The majority of clones sequenced were classified as Proteobacteria (Fig. 7). Partial sequencing suggested eight of 16 clones clustered with γ Proteobacteria sequences recovered from marine systems (Fig. 7), including marine sediments (Li *et al.*, 1999; Ravenschlag *et al.*, 1999) and shallow water hydrothermal vents (Sievert *et al.*, 2000). By in silico

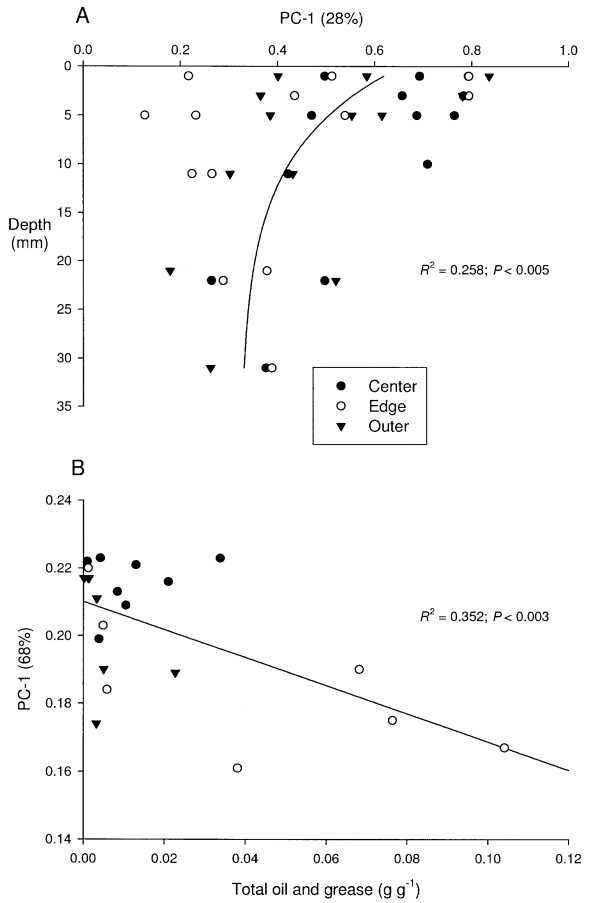

Fig. 5. Principal component (PC) of bacterial diversity versus depth (A) and total oil and grease (B). Principal components were calculated based on the number of peaks shared between *Hha* I-generated electropherograms and the relative heights of shared peaks (see *Experimental procedures*).
A. Locations (centre, edge and outer) correspond to locations relative to a seep mud volcano (see Fig. 2). The line represents an exponential decay model.
B. The principal component was recalculated after excluding data from samples collected from depths deeper than 6 mm. The line represents a linear model.

restriction we confirmed that these clones contained sequences consistent with *Hha* I TRFs between 189 and 213 bp long. Clone SBseep1 classified as Bacteroides (Fig. 7) and corresponded with the large peak observed at 94 bp in *Hha* I restrictions (Fig. 4). Clones that classified as alpha Proteobacteria and Planctomyces (Fig. 7) corresponded to peaks observed at 61 bp. Clones also showed similarity to representatives of Holophaga/Acidobacteria, Planctomyces, Actinobacteria and candidate division W3 that were previously recovered from marine systems (Fig. 7).

Discussion

Hydrocarbon seepage into the benthos of Santa Barbara Channel affects bacterial community structure (Fig. 5) and reduces bacterial diversity (Fig. 6). Although previous studies have shown changes in microbial communities as a result of oil spills (Lindstrom *et al*., 1999), this is the first study to look at the long-term effect of oil from naturally occurring seeps on sediment bacteria. Experimental additions to sediments suggest that many of the changes in microbial community structure due to oil addition are temporary (Macnaughton *et al*., 1999; Bachoon *et al*., 2001; Röling *et al*., 2002). For example, without nutrient addition, increases in bacterial biomass with hydrocarbon addition are ephemeral (Delille, 2000). Because sediments in the Coal Oil Point area have been exposed to hydrocarbons for centuries, there could be a large bacterial population adapted to oil-degradation or at least selected for oil tolerance at this site. Resource-ratio theory (Smith *et al*., 1998) predicts that changes in community structure would follow from changes in the ratio of limiting nutrients. In this marine seep region, the C/N ratio of sediments increased with oil content (Fig. 3). This shift in C/N ratio and the overall increase in carbon content with sediment hydrocarbon concentration (Fig. 3A) suggests that hydrocarbons have a profound effect on sediment quality. A shift in microbial community structure occurred with this shift in resource availability (Fig. 5). In applying resource-ratio theory we assume significant microbial consumption of hydrocarbons in seep sediments. This assumption follows from higher microbial biomass (Montagna *et al*., 1989) and activity (Montagna *et al*., 1986) in this seepage site as compared to surrounding environs.

Hydrocarbons may also indirectly control community structure and diversity by affecting the redox potential in sediments. Oxygen availability declines with depth in sediments, which results in a change in the electron acceptors utilized by microbes in carbon oxidation (Froelich

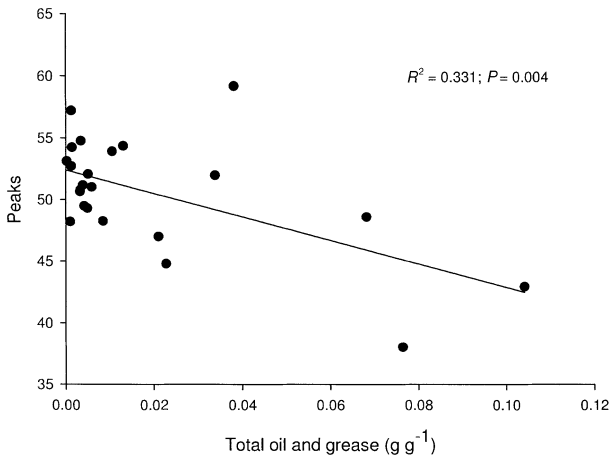

Fig. 6. Diversity estimates for surface sediments (0–6 mm) versus total oil and grease. Richness (number of peaks) was calculated from *Hha* I-generated TRFLPs.

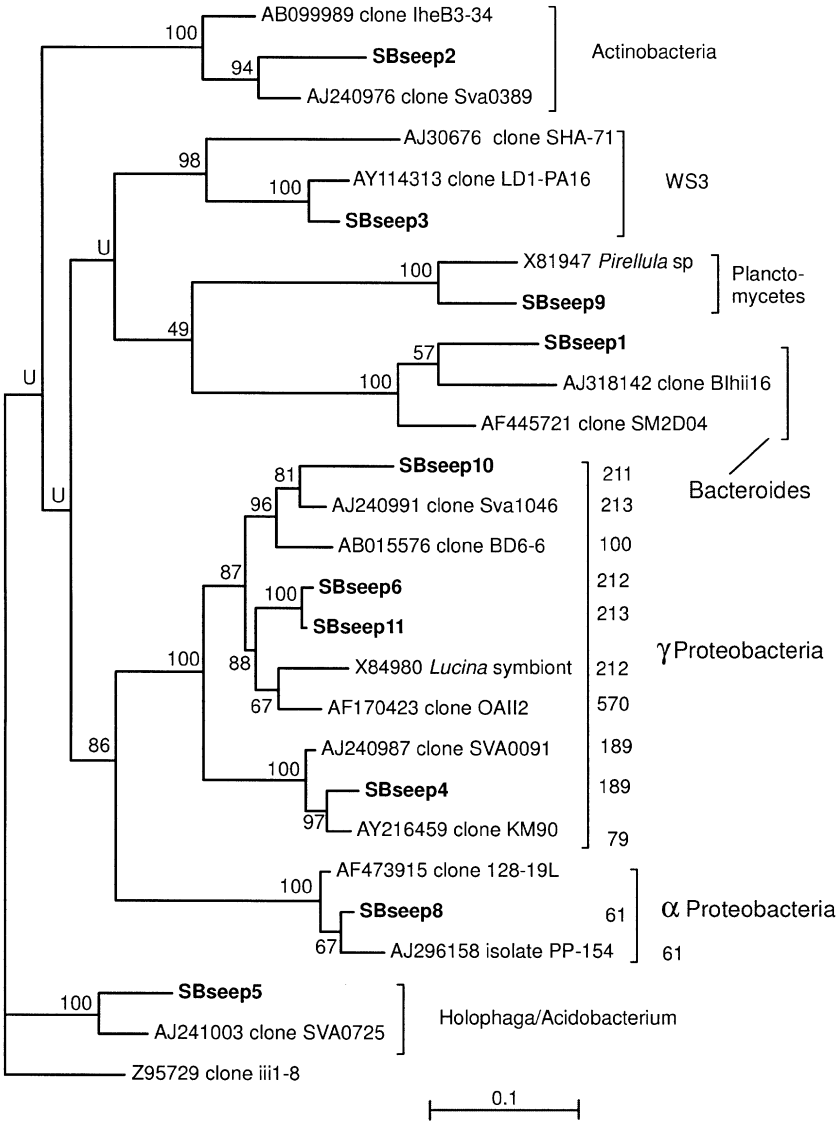

Fig. 7. Phylogenetic classification of clones. Clones sequenced in this study are in bold. Only five of 11 sequences that clustered with the Proteobacteria are shown. Alignments of 798 characters were used to generate the tree. The phenogram was drawn by the maximum likelihood method and bootstrap values were assigned to nodes as described previously (LaMontagne *et al*., 2003). 'U' indicates node not supported by bootstrap analysis. Scale represents a 10% difference in sequence. Predicted length of HhaI-generated TRFs, based on in silico restriction of clone sequences, is indicated to the right of the brackets.

et al., 1979) and presumably the species occupying these niches. In the Coal Oil Point field, the surface 2 cm are typically oxic, as evidenced by positive Eh values (Montagna *et al*., 1987), but these Eh values decrease with increasing sediment oil content (Montagna *et al*., 1987; Bauer *et al*., 1988). We observed that community structure changed below the sediment surface (Fig. 5A), which is consistent with an expectation that redox conditions and thus microbial communities change with depth. These changes were detected with PCR-TRFLP, which is susceptible to biases associated with DNA extraction and PCR-amplification; however, these biases appear relatively small when compared to variation in bacterial communities in natural systems (LaMontagne *et al*., 2003).

From macro-ecological species-area theory (Connor and McCoy, 1979), increasing the number of niches sampled will increase diversity estimates. For example, bacterial richness obtained from *Hha* I-generated TRFLPs in surface sediments outside the seepage area averaged approximately 80% of richness estimates obtained by *Hha* I-generated TRFLPs for surface sediments (0–2 cm) of Tokyo Bay (Urakawa *et al*., 2000). Larger sediment sections may contain several different habitats in terms of sediment Eh and other environmental factors. Richness estimates would tend to increase as these different niches are pooled during analysis. Similarly, the presence of oil, because of enhancement of sediment oxygen demand, would increase the rate of Eh change with depth. Thus, similar depth intervals could contain more, or if the sediments become completely anoxic, less niches.

Species-energy theory stems from species-area theory and postulates that systems with more energy contain

more biomass and more species (Wright, 1983). Because sediments contain orders of magnitude more microbial biomass than overlying waters (Schmidt *et al*., 1998), this theory can explain why the sediments studied herein contained two to three times more species than coastal waters sampled a few kilometres away (LaMontagne and Holden, 2003). However, biomass enhancement does not necessarily enhance diversity. In enclosed systems, addition of substrates tends to select for a community or an isolate capable of utilizing the added resource and thus reduces microbial diversity. For example, addition of inorganic nutrients and labile carbon (glucose) to seawater increases bacterial biomass but decreases bacterial diversity (Carlson *et al*., 2002). Theoretically, the heterogeneity of compounds in oil could support a diverse population of oil-degraders but few studies have examined the effect of substrate heterogeneity on microbial diversity in natural systems. Microbial diversity in the rhizosphere, as opposed to bulk soil (Brodie *et al*., 2002), appears to increase with diversity of above-ground plants (Kowalchuk *et al*., 2002) which suggests a link to plant-derived carbon. Further, a few species can dominate oil-amended mesocosms, even without nutrient addition (Röling *et al*., 2002).

Almost all of the clones screened in this study showed high similarity to yet to be cultured ribotypes previously recovered from marine sediments. These results suggest that marine sediments are distinctive habitats. Consistent with previous studies (Yakimov *et al*., 2002; Knittel *et al*., 2003), Proteobacteria dominated the sediment bacterial community studied herein. Hydrocarbons appeared to influence the structure of these sediment communities. Beyond serving as a carbon source, many hydrocarbons in oil are toxic to bacteria (Griffiths *et al*., 1981). Toxins can reduce diversity by eliminating species or enhance diversity by opening niches occupied by dominant species. The exponential decline in many peak heights with oil content suggests a toxicity effect. Based on in silico restriction of cloned rRNA genes, we hypothesize that ribotypes related to γ Proteobacteria were relatively sensitive to hydrocarbons. Similar to that observed in sediments that have not previously been exposed to hydrocarbons, the net effect of oil on microbes in the hydrocarbon seep area appears to reduce bacterial diversity. In this regard, bacterial diversity may indicate the exposure of sediment bacterial communities to hydrocarbons or to other toxic compounds.

Experimental procedures

Study site

Shane Seep (34°24.37′-N, 119°53.41′-W, Fig. 1), within the Coal Oil Point field, has been intensively investigated for several years (Leifer and Clark, 2002). The main sea surface features of Shane Seep in 2001 were two concentrated bubble plumes (~ 5 m diameter) originating from two east–west aligned hydrocarbon volcanoes with a common ridge. The plumes rise in a much larger and more dispersed bubble plume tens of meters in diameter (Leifer and Clark, 2002). The concentrated plumes are associated with strong (30 cm s^{-1}) upwelling flows (Leifer and Clark, 2002), whereas the more diffuse plume produces a weaker upwelling flow. The most active vents originated in hydrocarbon volcanoes rising about 1 m above the seabed (~ 22 m deep). The caldera floor was at roughly the same depth as the seabed. Volcano walls impede currents at this site.

Sediment sampling

Sediment samples were taken at three sites along a north–south transect from the mud volcano (Fig. 2): within the caldera (centre), on the flank of the caldera (edge) and about 13 m from the caldera (outer). SCUBA equipped divers collected three piston cores at each of these three sites in February 2001. Piston cores were constructed from 60 ml disposable polypropylene syringes (B-D, Franklin Lakes, NJ). Cores were transported upright and frozen (– 80°C) within 4 h of collection. Without allowing the frozen sediments to thaw, the cores were sectioned with a flame-sterilized razor at 2 mm intervals to a depth of 6 mm and then at 1 cm intervals to a depth of 3.6 cm. Core sections were homogenized and split into aliquots for microbial and sediment analysis and stored at −20°C. Replicate cores and core sections were treated independently for bacterial community characterization.

Nucleic acid analysis

Community DNA was extracted by bead-beating sediments (MoBio, Solana Beach, CA). DNA yield was quantified by Picogreen fluorometry as per manufacturer's instructions (Molecular Probes, Eugene, OR). Extracts were subsequently purified by size-exclusion, Sepharose spin columns (Jackson *et al*., 1997). Column purified DNA was precipitated by addition of isopropanol, resuspended in 10 mM Tris 1 mM EDTA buffer (pH 8) and quantified by UV-spectra. A_{260}/A_{230} ratios of purified DNA exceeded unity, which indicates sufficient purity for microbial community analysis with PCR-based techniques (LaMontagne *et al*., 2002). Small subunit (16S) ribosomal genes were PCR-amplified using universal eubacterial primers 8F hex (fluorescently labelled) and 1389R, then purified and digested with *Hha* I as described previously (LaMontagne and Holden, 2003). To determine the dependence of diversity estimates on restriction enzyme, a subset of the PCR-products were separately restricted with *Hae* III. The length of fluorescently labelled fragments was determined with an Applied Biosystems Instruments (Foster City, CA) model 3100 automated sequencer. Terminal restriction fragment length polymorphisms were aligned to determine the number of reproducible peaks. Fragments that deviated less than 0.5 bp in length were considered shared. Richness was calculated as the number of reproducible peaks. Shannon–Weaver diversity indices were calculated using peak heights as a metric of abundance as described previously (LaMontagne *et al*., 2002). Jaccard similarity coefficients

were calculated based on the number of peaks shared between *Hha* I-generated TRFLPs. Pearson correlation coefficients were calculated from log-transformed heights of the shared peaks. Principal components (PC) were reduced from a weighted matrix generated from the average of Jaccard and Pearson coefficients as described previously (LaMontagne *et al*., 2003).

To hypothesize the phylogeny of the oil-sensitive ribotypes, PCR products generated from two surface sections collected at the 'outside' site were restricted with *Msp* I, as previously described for *Hha* I and *Hae* III. Data from the three restriction enzymes were compared against an in silico restriction of published rRNA gene sequences with the program tRFLP Fragment Sorter (http://www.oardc.ohio-state.edu/trflpfragsort/). To generate a clone library, partial 16S rRNA genes were amplified from purified DNA extracted from the surface section at the 'outside' site. The primers 8F (no fluorescent label) and 907R were used as above and PCR products were cloned into the pCR®2.1 vector (Invitrogen, Carlsbad CA). Putative positive clones were selected by blue/white screening and restreaked to LB agar plates containing 75 μg ml^{-1} ampicilin. Plasmid DNA was obtained by standard methods and 16 clones were partially sequenced with the vector primers M13F and T7. Nucleotide sequences of clones were assigned GenBank accession numbers AY456979 to AY456989.

Sediment chemical analysis

Residual oil in the sediment cores was measured using a gravimetric procedure (Rhykerd *et al*., 1994). For each sample, 1 g of wet sediment was placed into a Teflon-lined screw cap tube and dried by mixing with anhydrous sodium sulphate. Dichloromethane (DCM) was added (2 ml) and the mixtures were shaken (250 r.p.m.) overnight at room temperature. The extracts were then filtered through no. 1 Whatman paper with additional sodium sulphate into preweighed test tubes. The sediment mixtures were extracted two additional times (2 ml DCM, shaken for 5 min) to enhance complete oil recovery, and filtered into test tubes as before. The extracts were evaporated using a stream of dry nitrogen and desiccated overnight. The final weight of the test tubes plus oil residue was recorded. Oil negative controls (sodium sulphate only) were also extracted. To determine total carbon and nitrogen, sediments were dried overnight at 70°C and analysed with a CEC 440HA Organic Elemental Analyzer (Exeter Analytical, North Chelmsford, MA). Chemicals (Fisher Scientific) were reagent grade or better.

Gas chromatography

A sediment sample was also analysed by gas chromatography using flame ionization detection (GC/FID). DCM extracts (above) were purified using an alumina column (150 mesh, chromatographic grade, combusted at 400°C for 4 h and stored at 120°C before use) and eluted with pentane for removal of matrix interferences. The purified extracts were concentrated to a final volume of 1.0 ml using a Kuderna-Danish evaporator. GC/FID of the extracts was performed using a Hewlett-Packard 5890 instrument fitted with a split/splitless injector (300°C) operated in splitless mode. Helium was the carrier gas; the column was fused silica (30-m long × 0.32-mm I.D.) coated with a DB-5 bonded phase (5%-phenyl 95% methylpolysiloxane, 0.25 μm film thickness). Initial oven temperature was 60°C (1 min), followed by an increase of 6°C min^{-1} to 300°C, where it was held for 10 min.

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